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p53 is a NF-Y- and p21-independent, Sp1-dependent repressor of cyclin B1 transcription

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Abstract The p53 tumour suppressor protein is a DNA-binding transcription factor activated in response to DNA damage. Inactivation of the p53 gene occurs in 40–60% of human tumours and a substantial body of work indicates that loss of p53 activity is a critical step in oncogenesis. p53 helps to protect against neoplasia by inducing death in cells that have sustained irreparable DNA damage or by blocking cell cycle progression to allow time for DNA repair. We have previously reported that p53 prevents G2/M transition by decreasing intracellular levels of cyclin B1 protein and attenuating the activity of the cyclin B1 promoter [S.A. Innocente, J.L. Abrahamson, J.P. Cogswell, J.M. Lee, p53 regulates a G2 checkpoint through cyclin B1, Proc. Natl. Acad. Sci. USA 96 (1999) 2147–2152]. The ability of p53 to control mitotic initiation by regulating intracellular cyclin B1 levels suggests that a cyclin B1-dependent G2 checkpoint has a role in preventing neoplastic transformation. Here, we show that p53-mediated attenuation of the cyclin B1 promoter occurs in both $p21^{+/+}$ and $p21^{-/-}$ cell lines. Furthermore, promoter attenuation is dependent on the presence of functional Sp1 binding sites and is independent of the NF-Y binding sites. We also find that wild-type, but not mutant, p53 protein binds Sp1 and the cyclin B1 promoter. This suggests that wild-type p53 mediates transcriptional repression of cyclin B1 through the Sp1 transcription factor.

Keywords: Transcription repression; p53; Sp1; Cyclin B1; Cell cycle

1. Introduction

The eukaryotic cell cycle consists of sequential rounds of chromosomal DNA synthesis (S) and chromosomal segregation (M) separated by G1 and G2 gap phases. Transition between cell cycle boundaries is under the control of the cyclin family of proteins and the cyclin-dependent kinases (CDKs). Mammalian cells have evolved regulatory mechanisms, termed checkpoints, which prevent cell cycle transition after exposure to DNA damage or other cellular stress [2]. Multiple, independent checkpoints have been identified that prevent G1/S and G2/M transition after exposure to DNA damage [3]. Checkpoint inactivation is a property of most, if not all, cancer cells [4].

Mitotic entry is under the control of the B-type cyclins [5]. Cyclin B functions by binding to the cdc2 kinase; the resultant complex is termed the maturation promoting factor (MPF). Cdc2 is non-functional without its cyclin B partner. Control of cyclin B1/cdc2 abundance and activity at multiple levels ensures correct mitotic timing [6]. Cdc2 kinase activity is inhibited by phosphorylation at Y15 and T14 residues; wee1 phosphorylates and cdc25C dephosphorylates these residues [7]. The human *cyclin B1* gene is initially activated in S-phase and its activity peaks at G2/M [8]. The human cyclin B1 promoter contains several *cis*-elements that allow cell cycle-specific expression. These elements include binding sites for the transcription factors USF [9], NF-Y [10], MyoD, Ap-2 and Sp1 [11]. We have previously shown that the p53 tumour suppressor activates a G2/M checkpoint by repressing cyclin B1 promoter activity [1]. However, the mechanism responsible remains to be full elucidated. The p53 protein is a transcription factor that binds to DNA in a sequence-specific fashion to activate transcription of target genes. The consensus DNA-binding sequence for p53 consists of two repeats of the 10 bp motif 5′-PuPuPuC(A/T)(A/T)GPyPyPy-3′ separated by 0–13 bp [12]. Activation of p53 by cellular stresses such as DNA damage, hypoxia and oncogene activation leads to the induction of cell cycle arrest or apoptosis. Transactivation of the p53 target genes p21, GADD45 and 14-3-3 is involved in mediating cell cycle arrest by p53 [13–16]. The p21 protein inhibits DNA synthesis by inactivating the CDKs necessary for G1/S transition. Up regulation of 14-3-3σ by p53 is thought to inhibit mitosis by attenuating the nuclear transport of cyclin B1 [17].

In addition to activating transcription, p53 is also able to repress transcription from various promoters. Transcriptional repression by p53 has been shown be important for the promotion of apoptosis [18]. Targets of p53 repression include *cyclin B1*, *β-tubulin*, *Map4*, *survivin*, *AFP* and *POLD1* genes [1,19–23]. Here, we report that p53 can repress cyclin B1 transcription through the transcriptional activator Sp1. Mutation of both the 5′ and 3′ Sp1 binding sites in the cyclin B1 promoter abrogated the repression effect of p53, whereas mutation of both the 3′ and 5′ NF-Y binding sites had little effect. We also found that wild-type, but not mutant, p53 interacts with Sp1 and the cyclin B1 promoter. These observations are consistent with the idea that

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p53-mediated repression of cyclin B1 requires interaction of p53 with Sp1 bound to the cyclin B1 promoter.

2. Materials and methods

2.1. Cell culture and cell lines

Saos-2 and SKOV-3 were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in McCoy's 5A medium supplemented with 10% FBS and 2% antibiotic–antimycotic (Invitrogen, Burlington, ON). The HCT-116 ($p21^{+/+}$) and HCT-116 ($p21^{-/-}$) were a gift of Dr. Bill Taylor (The Cleveland Clinic, Cleveland, OH) with permission from Dr. Bert Vogelstein (Howard Hughes Medical Institute, Baltimore, MD). HCT-116 cells were maintained in DMEM with 10% FBS and 2% antibiotic–antimycotic. Ts-SKOV3 was a kind gift of Dr. Massimo Broggini and is a derivative of the parental SKOV3 ovarian cancer line and details of its construction are found elsewhere [24]. For recombinant protein production, Sf9 cells (*Spodoptera frugiperda*, ATCC) were grown in Grace's Insect Medium with L-glutamine (Invitrogen) and supplemented with 10% yeastolate and 10% heat-inactivated FBS and cultured at 28 °C.

2.2. Plasmid constructs

The 1050 and 287 cyclin B1 promoter sequences were gifts of Drs. John Cogswell (Glaxo Research Institute, NC) and Karen Katula (University of North Carolina, Greensboro, NC), respectively, and have been described previously [9,10]. The 1050 and 287 luciferase promoter reporter construct were created by cloning either the 1050 or 287 fragments into the *Kpn*I and *Hin*dIII sites in the pGL3-Enhancer luciferase reporter vector (Promega, Madison, WI). The Sp1 binding site mutants (287cycB1-3′-Sp-1, 287cycB1-5′-Sp-1, and cycB1-3/5-Sp-1) were generated via PCR using the following primers 5′-Sp1-Mut-R (5′-GTGCGCCCAACACCTGG-3′) and 3′-Sp1-Mut-R (5′-CGAGAGGTTGGGGGCCA-3′). The p53 expression plasmid was derived by cloning the human wild-type p53 cDNA into the *Xba*I/*Eco*RI sites in pCDNA3 (Invitrogen) and was a kind gift of Dr. Carol Prives (Columbia University, New York, NY). The Sp1 expression plasmid was a kind gift of Dr. Anil Rustgi (University of Pennsylvania, Philadelphia, PA) and its construction has been described elsewhere [25]. The sequences of all plasmid constructs were verified by automated DNA sequencing by the Institute of Molecular Biology (MOBIX) at McMaster University.

2.3. Transient transfection and luciferase reporter gene assays

SKOV-3 and Saos-2 cells were seeded at 1.5×10^5 cells per well in 6-well plates and grown overnight in growth medium. Cells were transfected with 1 μg of p53 expression plasmid or control pcDNA3 plasmid and 3 μg of pGL3 reporter constructs using GenePorter I (Gene Therapy Systems, San Diego, CA) according to the manufacturer's protocol at a DNA/reagent ratio of 1:5. Three hours post-transfection, the medium was changed and fresh growth media was added. For luciferase assays, cells transfected in six-well plates were washed three times with 2 ml of ice-cold PBS. Cells were lysed in 200 μl of lysis buffer (1% (vol/vol) Triton X-100, 25 mM glyclglycine, pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA, and 1 mM DTT). Samples were assayed in a Tropix TR717 multiwell luminometer using 70 μl cell lysate, 175 μl assay buffer (25 mM glyclglycine, pH 7.8, 15 mM $MgSo_4$, 4 mM EGTA, 2 mM ATP, and 1 mM DTT) and 140 μl of luciferin (1 mM luciferin in 25 mM glyclglycine, pH 7.8, and 10 mM DTT). Luciferase activity was measured a 10 s delay and 20 s integration time and normalized to the protein concentration of each sample.

2.4. Western blotting and immunoprecipitation

Cells were lysed in RIPA (25 mM Tris, pH 8.2, 50 mM NaCl, 0.1% SDS, 0.5% Nonidet P-40 and 0.5% deoxycholate) buffer with protease inhibitors. Proteins were resolved on SDS–PAGE gels and transferred to nitrocellulose via semi-dry transfer (BioRad). Immunoblots were blocked in 5% milk in TBST for 1 h and washed three times with fresh TBST. Primary antibodies were incubated with immunoblots for 1 h at room temperature and washed three times with fresh TBST. Similar concentrations of secondary antibodies were incubated with immunoblots for an additional hour at room temperature. Proteins were detected using ECL-plus (Amersham Scientific) and a phosphorimager (GeneStorm 860, Molecular Dynamics). The p53 primary antibody PAb 246 and the p53 Western Blotting kit were purchased from EMD Biosciences (San Diego, CA). The Sp1 monoclonal antibody E-3 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). For immunoprecipitations, 100 μg of whole cell lysate was incubated with 2 μg of antibody at 4 °C with gentle rocking overnight. 20 μl (packed volume) of protein G Sepharose beads (Invitrogen) were added and samples were incubated at 4 °C with gentle rocking for 1 h. Immunoprecipitates were collected via centrifugation and washed three times with lysis buffer. Samples were resuspended in 2× loading buffer, boiled for 5 min and subjected to SDS–PAGE.

2.5. Chromatin immunoprecipitation assays

Ts-SKOV-3 cells were grown in 150 mm plates and then transferred to a 32 °C incubator for 12–14 h. Cells were cross-linked with formaldehyde (1%) for 10 min on a rocking platform at room temperature. Cross-linking was stopped following the addition of glycine (final concentration of 125 mM) at room temperature for 5 min. Cells were washed three times with ice-cold PBS and then scraped into 1 ml of PBS containing protease inhibitors and pelleted at $700 \times g$. Cell pellets were resuspended in 1 ml lysis buffer (5 mM PIPES, pH 8.0, 85 mM KCl, and 0.5% NP-40) with protease inhibitors and incubated on ice for 10 min. Nuclei were pelleted and resuspended in nuclear lysis buffer (50 mM Tris, pH 8.1, 10 mM EDTA, and 1% SDS) with protease inhibitors and incubated on ice for 10 min. Chromatin was sonicated for a total of 3 min per sample (20 s bursts followed by 1 min on ice) in a Branson Sonifier 250 with a 5 mm microtip. Samples were diluted fivefold in dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris, pH 8.1, and 16 mM NaCl with protease inhibitors). Samples were pre-cleared with 60 μl of protein A agarose for 1 h at 4 °C. Samples were incubated with of either 5 μg of p53 PAb 421 (Oncogene Science) or Sp1 E-3 (Santa Cruz Biotechnology) or with no antibody overnight at 4 °C. Immune complexes were collected with 60 μl of protein A and washed once with 1 ml low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris, pH 8.1, and 150 mM NaCl), once with 1 ml high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris, pH 8.1, and 500 mM NaCl), once with 1 ml LiCl buffer (0.25 mM LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, and 10 mM Tris, pH 8.0) and twice with 1 ml TE buffer. Samples were then subjected to PCR. Samples were eluted with fresh elution buffer (1% SDS, 0.1 M $NaHCO_3$), DNA-protein cross-links were reversed by incubation at 65 °C and 5 M salt. Proteins were digested with proteinase K and DNA was purified using QIAquick spin columns as per manufacturer's protocol. DNA was analyzed via PCR using the following primers: ChIP forward 5′-AGAGGCAGACCACGTGAGA-3′ and ChIP reverse 5′-TTCCTCTTCACCAGGCAGCA-3′.

2.6. Electrophoretic mobility shift assays

EMSA assays were conducted as follows, 2 μl of 5× EMSA buffer (20% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris–Cl, pH 7.5, and 0.25 mg/ml poly (dI-dC)), 25 ng of 5′-labeled 287 cyclin B1 probe, 300 ng of recombinant human Sp1 or p53 protein (Promega) and dH_2O to 10 μl. Reactions were incubated at room temperature for 30 min and samples to be super shifted were incubated a further 30 min with 5 μg of anti-Sp1 (E6, Santa Cruz Biotechnology) or anti-p53 (PAb 421, Oncogene Science) antibody. Samples were then carefully loaded onto a pre-run 0.5× TBE 4% native PAGE gel with an 80:1 (acrylamide:bisacrylamide) ratio. Flanking lanes of bromophenol blue loading dye was loaded onto the gel to monitor progress. The gel was run at a constant current of 25 mA until the bromophenol blue dye-front was three quarters down the gel. The gel was subsequently dried and exposed to a phosphorimager screen.

3. Results

3.1. The human cyclin B1 promoter is negatively regulated in a p53-dependent manner

To determine the effect of ectopic expression of p53 on transcription of the human cyclin B1 promoter, we transiently transfected the p53-null cell lines Saos-2 and SKOV3 with

human cyclin B1 promoter luciferase vectors pGL3-1050 wt and pGL3-287 wt and an expression vector for wild-type human p53 (pcDNA3-p53 wt). We have previously used both Saos-2 and SKOV3 to study transcriptional repression by p53 [1]. The pGL3-1050 wt and pGL3-287 wt reporter constructs contain the first 1050 and 287 bp, respectively, of the human cyclin B1 promoter 5′ to the transcriptional start site as determined by Cogswell et al. [9]. As shown in Fig. 1A and B, ectopic expression of wild-type p53 decreases the activity of both the −1050 and −287 cyclin B1 promoter fragments in SKOV3 and Saos-2 cell lines. In SKOV3, p53 repressed 1050- and 287 promoter activity 20-fold and 10-fold, respectively. In Saos-2, p53 repressed 1050 activity threefold, and 287 fivefold. Repression of both the 1050 and 287 constructs by wild-type p53 indicate that the *cis*-repressible elements must lie within the 287 region.

3.2. p53-dependent repression of cyclin B1 transcription does not require p21

Previously, it has been shown that induction of p21 was required for p53-mediated repression of cdc2 transcription [26]. To determine whether p21 was required for repression of cyclin B1, we used the colorectal carcinoma cell line HCT-116 that expresses p21 (HCT116-p21$^{+/+}$) and a variant that has no p21 (HCT116-p21$^{-/-}$). As shown in Fig. 1C, the presence or absence of p21 expression had no effect on p53-mediated transcriptional repression of cyclin B1 (Fig. 1C). In both cell lines, p53 inhibited 287 promoter activity 2.5-fold. While the ability of p53 to repress cyclin B1 is reduced in HCT-116 compared to SKOV3 and Saos2, the magnitude of repression is not changed between the p21+ and p21− HCT-116 variants. This suggests that the p53-mediated transcriptional repression of the cyclin B1 promoter is independent of p21. Moreover, Saos-2 cells have no Rb [27] and therefore repression of cyclin B1 by p53 is also independent of Rb.

3.3. Mutation of the CCAAT boxes has no effect on p53-mediated inhibition of cyclin B1 transcription

Induction of cyclin B1 transcription in the G2 phase of the cell cycle is dependent in part on the binding of the NF-Y transcription factor to the CCAAT sequence within the promoter [10]. It has previously been reported that mutation of NF-Y binding sites in the cyclin B1 promoter ablated p53-mediated transcriptional repression senescing human fibroblasts [28].

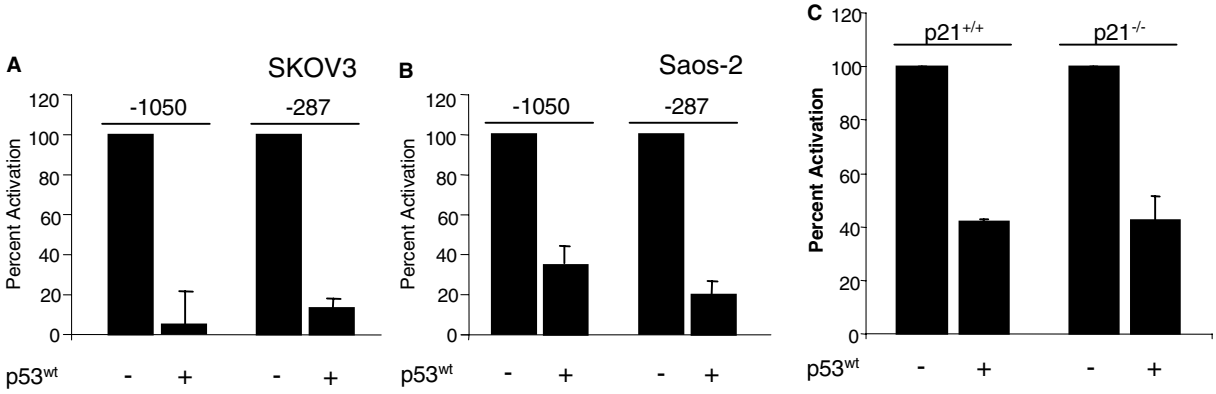

Fig. 1. p53 is a p21-independent repressor of cyclin B1 transcription. (A) Ectopic expression of p53 in the p53-null cells SKOV3 and Saos-2 represses cyclin B1 promoter activity. p53 is able to repress both the −1050 and −287 cyclin B1 promoter fragments in both SKOV3 (left) and Saos-2 (right). (B) p53-mediated cyclin B1 transcriptional repression is not dependent on p21. Ectopic expression of p53 in either p21-positive or p21-negative HCT-116 colon cancer cells results in transcriptional repression of cyclin B1 activity. All data points are expressed as percent activation compared to the pcDNA3 empty vector control transfection and represent the mean of three independent experiments together with standard deviation.

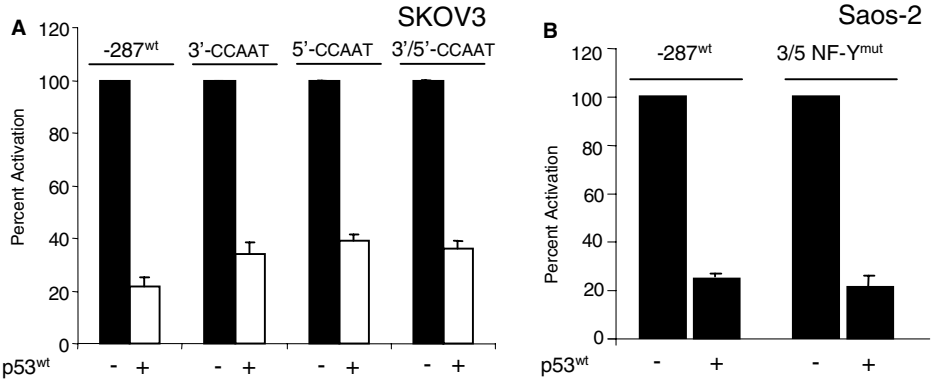

Fig. 2. Repression of cyclin B1 promoter activity is independent of NF-Y sites. (A) p53 can repress the transcription of the 287 cyclin B1 luciferase and the 3′, 5′ and 3′/5′ NF-Y mutant cyclin B1 constructs in SKOV3 cells. (B) p53 can repress the activity of cyclin B1 287 promoters lacking both the 3′ and 5′-NF-Y sites in Saos-2 cells. All data points are expressed as percent activation compared to the pcDNA3 empty vector control transfection and represent the mean of three independent experiments together with standard deviation.

To determine whether NF-Y was important in our system, we tested cyclin B1 promoter variants lacking both identified NF-Y binding sites. As shown in Fig. 2A and B, overall levels of p53-dependent repression of the promoter remained the same between the wild-type promoter and the NF-Y single (3′CCAAT or 5/CCAAT) or double (3′/5′ CCAAT) mutants. All constructs were repressed approximately 4–5-fold in both SKOV3 and Saos-2 cells. p53 expression levels were similar between transfections as confirmed by Western blot (not shown). Since mutation of both the CCAAT boxes in the human cyclin B1 promoter have no effect on transcriptional repression by p53, we conclude that NF-Y is not involved in p53-mediated control of cyclin B1 promoter activity.

3.4. Importance of Sp1 in transcriptional repression by wild-type p53

The human cyclin B1 promoter contains a number of transcription factor binding sites in addition to NF-Y, among them USF, MyoD1 sites, an E2F site and Sp1 sites [29]. To determine whether Sp1 was involved, we mutated Sp1 binding sites. Sp1 binds to its consensus sequence 5′-GGCGG-3′ and there are two such motifs in the cyclin B1 promoter, one beginning at −259 and another at −140, relative to transcription start. These sites were mutated singly and in tandem. As shown in Fig. 3A, mutation of either the 3′- or the 5′-Sp1 sites resulted in constructs that were still repressible by wild-type p53. Each of the single mutants was repressed 4–5-fold by

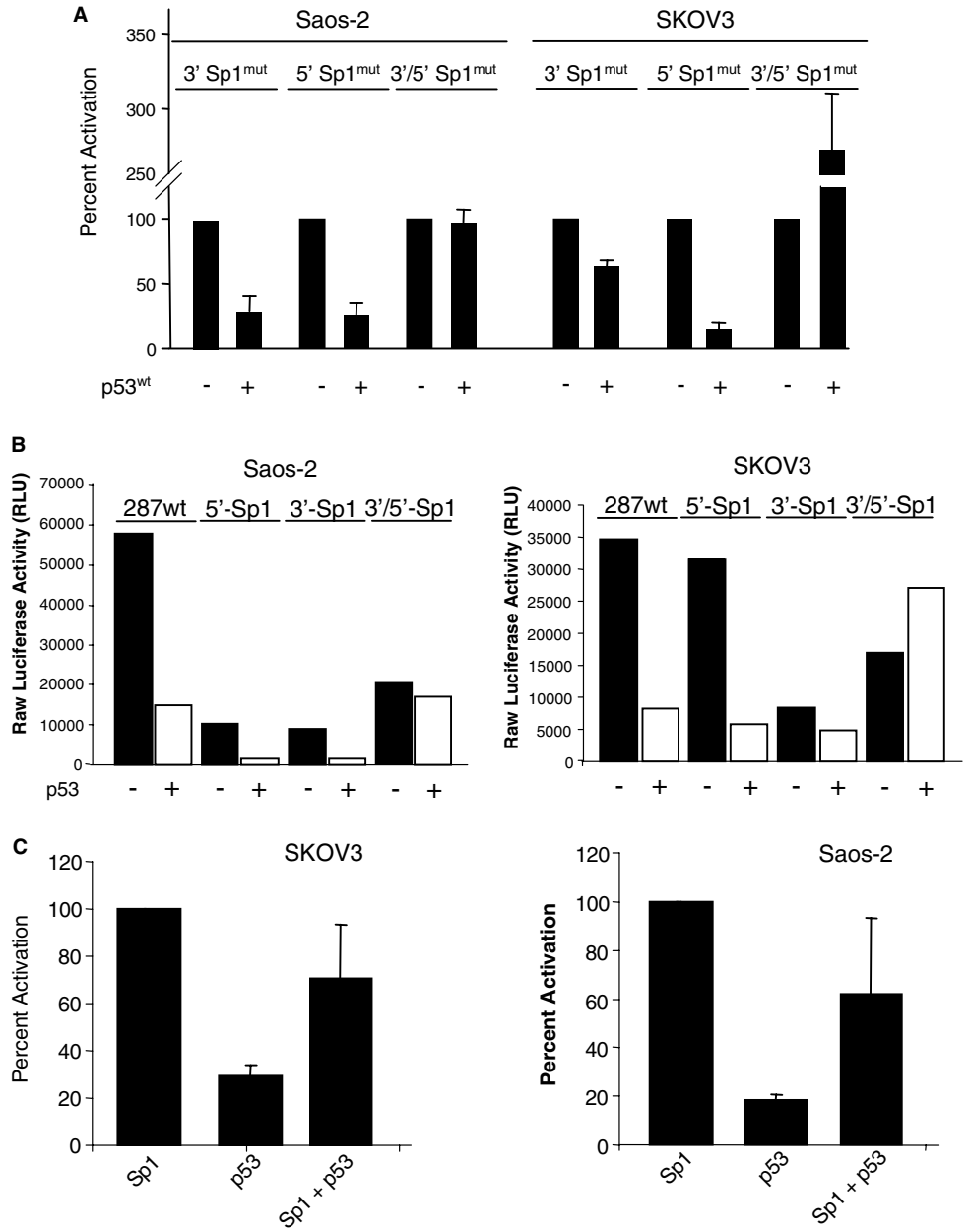

Fig. 3. p53-mediated repression of cyclin B1 transcription is dependent on functional Sp1 transcription factor binding sites. (A) Mutation of proximal and distal Sp1 sites abrogates p53-mediated transcriptional repression of the cyclin B1 promoter in both Saos-2 (left) and SKOV3 (right) cells. All data points are expressed as percent activation compared to the pcDNA3 empty vector control transfection and represent the mean of at least three independent experiments together with standard deviation. (B) A representative co-transfection experiment showing absolute luciferase values. (C) Expression of Sp1 can rescue the p53-mediated transcriptional repression of the cyclin B1 promoter in SKOV3 and Saos-2 cells.

p53, similar to wild-type. On the other hand, mutation of both the 3′ and 5′ Sp1 sites resulted in complete abrogation of repression (Fig. 3A). As shown in a single representative transient co-transfection experiment (Fig. 3B), Sp1 mutation reduced absolute activity of the construct as measured by raw luciferase activity, but this reduction was still substantially above background to detect p53-dependent transcription repression. We observed an increase in p53-dependent activity of the double Sp1 mutant in SKOV3 cells. The mechanism for this is currently unknown. To further clarify and extend these observations, we next determined whether ectopic expression of Sp1 could affect p53-mediated repression. As shown in Fig. 3B and C, expression of Sp1 in the presence of p53 allowed for nearly wild-type cyclin B1 promoter activity. These results suggest that Sp1 may have an important role in p53-mediated control of cyclin B1 transcription.

3.5. p53 interacts with Sp1 in vivo

We next investigated whether p53 could bind to Sp1. To that end, we infected Sf9 cells with baculovirus expressing human p53 and Sp1 constructs. As shown in Fig. 4A, wild-type p53 and Sp1 reciprocally co-immunoprecipitate in Sf-9 cells (Fig. 4A). To test whether Sp1 binds to either wild-type or mutant p53, can measured p53/Sp1 co-immunoprecipitation in the Ts-SKOV3 cell line. Ts-SKOV3 is a human cell line that stably expresses a copy of the mouse p53 mutant allele $p53^{val135}$. At 37 °C the $p53^{val135}$ protein has a mutant conformation but adopts a wild-type conformation and function at 32 °C [24]. Using Ts-SKOV3, we find that there is little Sp1/p53 interaction when p53 is in its mutant conformation (37 °C) but there is substantial interaction with both the 95 and 106 kDa forms of Sp1 when p53 is in the wild-type (32 °C) conformation (Fig. 4B). There are no detectable changes in the levels of p53 protein in cells grown at 32 °C versus 37 °C (Fig. 4B, bottom). Thus, both p53 and Sp1 physically interact but this interaction preferentially occurs with wild-type p53.

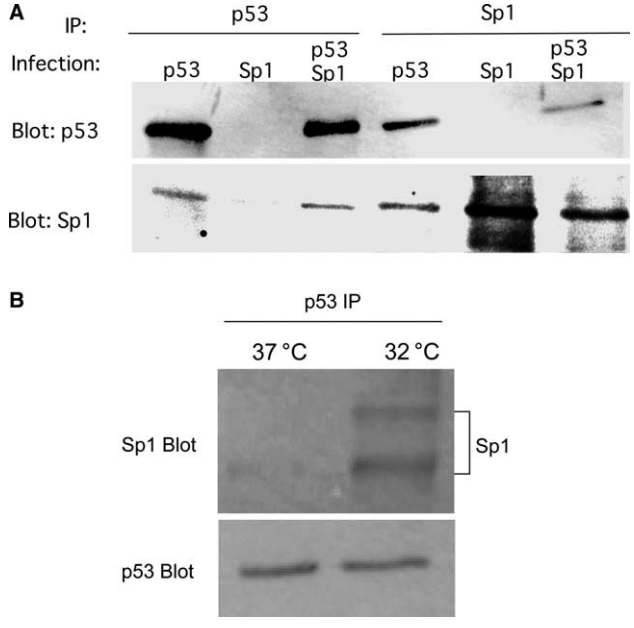

Fig. 4. p53 and Sp1 interact in vivo. (A) Baculovirus-expressed human p53 and Sp1 immunoprecipitate in *Sf*9 cells. (B) In Ts-SKOV3 cells grown at 32 °C, wild-type p53 co-immunoprecipitates strongly with both the 95 and 106 kDa Sp1 proteins at 32 °C and only very weakly at 37 °C (top panel). p53 protein can be detected in the Ts-SKOV3 cells at either 32 or 37 °C (lower panel).

3.6. p53 and Sp1 can interact with the human cyclin B1 promoter

To test if p53 and Sp1 can interact with the cyclin B1 promoter in vivo, we conducted chromatin immunoprecipitation (ChIP) assays using the Ts-SKOV3 cells described above. PCR primers were designed to specifically amplify the region of the human *cyclin B1* promoter containing both Sp1 DNA-binding sites. As shown in Fig. 5, Sp1 is found on the cyclin B1 promoter at both 37 and 32 °C temperatures (Fig. 5). This suggests that the switch from the mutant to the wild-type p53 conformation between 37 and 32 °C does not result in Sp1 dissociation from the cyclin B1 promoter. On the other hand, p53 is interacts with the cyclin B1 promoter at 32 °C but is only poorly detected at 37 °C. The low levels of cyclin B1 promoter PCR products at 37 °C is most likely a result of cross-linking at 23 °C and the high sensitivity of the ChIP technique. These data indicates that p53 interacts with the cyclin B1 promoter primarily in its wild-type conformation.

To determine whether p53 directly bound the cyclin B1 promoter, we used EMSA assays to measure direct binding of p53 and Sp1 to the 287-promoter fragment. As shown in Fig. 5B, the Sp1 protein can interact with the 287 fragment, as measured by a retardation of 287 mobility. A Sp1 antibody further

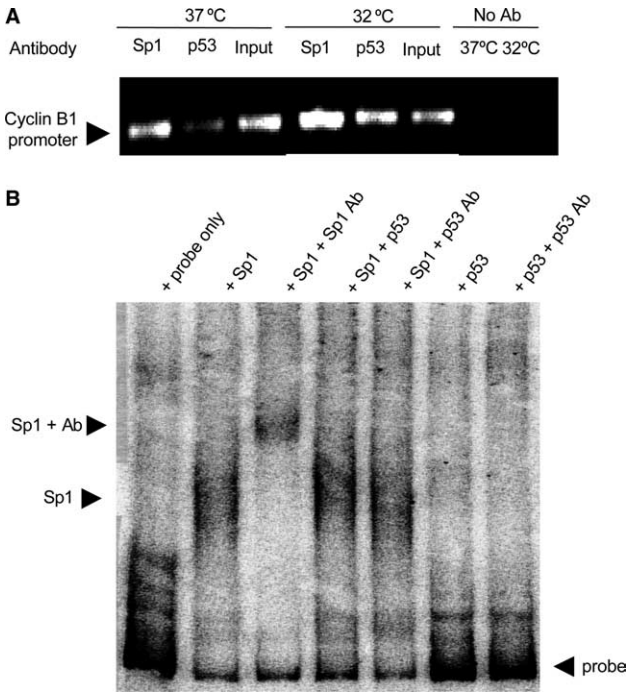

Fig. 5. p53 and Sp1 interact on the cyclin B1 promoter. (A) Sp1 protein can immunoprecipitate the cyclin B1 promoter at either 32 or 37 °C, whereas p53 immunoprecipitate the cyclin B1 promoter very weakly at 37 °C (mutant conformation) and strongly at 32 °C (wild-type conformation). The total chromatin input (1:300 dilution) control was used as a positive control. The no antibody control showed no bands at either 37 or 32 °C. (B) Sp1, but not p53 interact directly with the 287 promoter fragment in an EMSA assay. Sp1 retards the mobility of the 287 probe and this retardation can be increased with a Sp1 antibody. Neither p53 protein nor p53 antibody retards 287 mobility with or without Sp1.

decreases electrophoretic mobility. On the other hand, p53 does not detectably interact with the 287 promoter, nor does it interfere with Sp1 biding. This suggests that the p53 protein does bind the cyclin B1 promoter directly. Furthermore, there is no consensus p53 binding site within the cyclin B1 promoter. Since we observe that only wild-type p53 interacts with Sp1, our data is consistent with a model where p53 inhibits cyclin B1 transcription by interacting with promoter-bound Sp1 rather than the promoter itself, thereby blocking its ability to transactivate cyclin B1.

4. Discussion

In this report, we have investigated the role of the tumour suppressor protein p53 in regulating cyclin B1 expression. We find that p53-mediated repression of the cyclin B1 promoter is dependent on Sp1 binding sites and that Sp1 expression can rescue the p53-dependent cyclin B1 repression. Furthermore, Sp1 can co-immunoprecipitate with wild-type, but not mutant, p53. p53-mediated repression of cyclin B1 is independent of p21 and NF-Y. Thus, our data are consistent with the idea that p53-mediated repression of cyclin B1 is likely to involve Sp1.

p53 has previously been shown to repress cyclin B1 transcription in several cell types [30–32]. Taylor et al. have proposed that p53 can repress cdc2 transcription via the induction of p21WAF1 which in turn enhances the binding of p130 and E2F4 to the cdc2 promoter to repress it. In our hands, p53 can repress cyclin B1 transcription in both $p21^{+/+}$ and $p21^{-/-}$ cells, indicates that p21 is not absolutely required for cyclin B1 repression. It is likely, therefore, that there are p21-dependent and -independent pathways of repression.

In this report, we find that NF-Y does not seem to be critical in repression of cyclin B1 as mutation of both CCAAT sites in the cyclin B1 promoter still allowed the construct to be repressed p53 in both SKOV3 and Saos-2 cells. This issue is controversial as other reports suggest NF-Y may be important for p53-mediated cyclin B1 repression during senescence [28,30]. However, other data from Taylor et al. [29] have shown that p53-mediated repression of cyclin B1 requires regions −287 to −123 of the promoter. This region does not contain any NF-Y binding sites but does contain the distal Sp1 site, lending further support to our idea that Sp1 and not NF-Y is the important transcriptional activator mediating cyclin B1 transcriptional repression by p53.

Our results have shown that the mutation of the transcriptional activator Sp1 binding sites in the cyclin B1 promoter completely abolished the p53-mediated repression of cyclin B1 transcription. This repression requires the co-operation of both the proximal and distal Sp1 sites as mutation of either Sp1 site alone still conferred repression by p53. p53 has previously been shown to repress transcription from the HIV-LTR [33], the *POLD*1 [23] and *hTERT* [34,35] genes through Sp1. As there are no consensus p53-binding sites in the human cyclin B1 promoter and we were unable to detect p53 interaction using an EMSA assay, it seems unlikely that p53 mediates repression through direct DNA binding. We have shown that wild-type p53 can immunoprecipitate with Sp1 and that both proteins can be detected in the vicinity of the cyclin B1 promoter. Mutant p53 does not interact with the Sp1 protein and interacts very poorly with the cyclin B1 promoter. This suggests that p53 mediates cyclin B1 repression through a protein-protein interaction with DNA-bound Sp1 protein (Fig. 6). It is possible that this p53–Sp1 interaction interferes with the downstream recruitment of the TFIID general transcriptional machinery complex or other transcriptional activators. Thus, our results show that p53 can function as a Sp1-dependent inhibitor of cyclin B1 transcription, suggesting an important role for p53 tumour development.

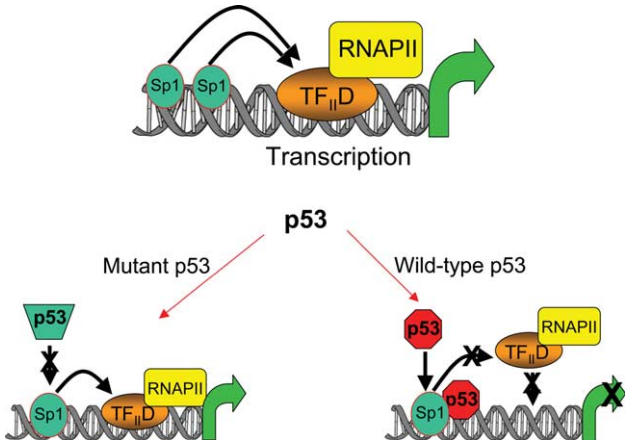

Fig. 6. Hypothetical mechanism for Sp1-dependant p53-mediated cyclin B1 transcriptional repression. p53 interacts with the DNA-bound transcriptional activator Sp1 and interferes with the recruitment of the general transcriptional machinery and thus, results in repression of cyclin B1 transcription.

Sp1 can function as a dual activator and repressor of transcription. As such, interaction between p53 and Sp1 does not always lead to transcriptional repression. It has previously been reported that interaction between p53 and Sp1 leads to activation of p21 gene transcription [36]. In the case of p21 transactivation, p53 binding to Sp1 displaces HDAC1 from Sp1 and increases p21 promoter activity concomitant with histone hyper-acetylation at the p21 promoter [37]. Conversely, transcriptional repression can be achieved by disconnecting communication between Sp1 and the general transcriptional machinery [38]. We postulate that p53 interferes with Sp1-dependent recruitment of the general transcriptional machinery to the cyclin B1 promoter and therefore reduces the levels of cyclin B1 transcription. Alternatively, it is possible that the p53–Sp1 complex could be a signal for the recruitment of repressor complexes, including histone deacetylases, to the cyclin B1 promoter to repress transcription. Further characterization of the mechanism by which Sp1 regulates cyclin B1 transcription will be necessary to address this issue.

The overexpression of cyclin B1 in a number of carcinomas [39–42] raises the possibility that a failure to control cyclin B1 promoter activity by p53 may be a prerequisite for tumour growth. Future work in our lab will further investigate the mechanisms involved in the p53-mediated repression of cyclin B1 transcription.

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